of Law will be entered on the same date herewith.

ORDER AND JUDGMENT

In accordance with the Findings of Fact and Conclusions of Law entered on the same date herewith,

IT IS HEREBY ORDERED AND ADJUDGED, as follows:

1. The Nolan patent (No. 4,506,189), issued on March 19, 1985, is a valid patent.

2. By the manufacture, production, sale and distribution of its SAF-T-COTE fluorescent lamp, Trojan has infringed the Nolan patent.

3. By virtue of this infringement, Shat-R-Shield is entitled to injunctive relief. Trojan shall immediately cease and desist from the manufacture, production, sale and distribution of the SAF-T-COTE fluorescent lamp.

4. Trojan shall recall all the SAF-T-COTE fluorescent lamps sold to and still in the possession of its customers.

5. The Court having determined that Trojan's infringement was not willful and wanton, Shat-R-Shield is not entitled to treble damages.

6. Shat-R-Shield shall have no accounting for monetary damages.

7. The Court having found that this is not an exceptional case, Shat-R-Shield is not entitled to its attorney's fees.

8. All claims having been resolved as to all parties herein, this action is now DISMISSED and STRICKEN from the docket.

9. There being no just reason for delay, this is a FINAL and APPEALABLE Order and Judgment.

Court of Appeals, Federal Circuit

In re Wands

No. 87-1454

Decided September 30, 1988

PATENTS

1. Patentability/Validity — Adequacy of disclosure (§115.12)

Data disclosed in application for immunoassay method patent, which shows that applicants screened nine of 143 cell lines developed for production of antibody necessary to practice invention, stored remainder of said cell lines, and found that four out of nine cell lines screened produced antibody falling within limitation of claims, were erroneously interpreted by Board of Patent Appeals and Interferences as failing to meet disclosure requirements of 35 USC 112, since board's characterization of stored cell lines as "failures" demonstrating unreliability of applicants' methods was improper in view of fact that such unscreened cell lines prove nothing concerning probability of success of person skilled in art attempting to obtain requisite antibodies using applicants' methods.

2. Patentability/Validity — Adequacy of disclosure (§115.12)

Disclosure in application for immunoassay method patent does not fail to meet enablement requirement of 35 USC 112 by requiring "undue experimentation," even though production of monoclonal antibodies necessary to practice invention first requires production and screening of numerous antibody producing cells or "hybridomas," since practitioners of art are prepared to screen negative hybridomas in order to find those that produce desired antibodies, since in monoclonal antibody art one "experiment" is not simply screening of one hybridoma but rather is entire attempt to make desired antibody, and since record indicates that amount of effort needed to obtain desired antibodies is not excessive, in view of applicants' success in each attempt to produce antibody that satisfied all claim limitations.

Appeal from decision of Patent and Trademark Office, Board of Patent Appeals and Interferences.

Application for patent of Jack R. Wands, Vincent R. Zurawski, Jr., and Hubert J. P. Schoemaker, serial number 188,735. From decision of Board of Patent Appeals and Interferences affirming rejection of application, applicants appeal. Reversed; Newman, J., concurring in part and dissenting in part in separate opinion.

Jorge A. Goldstein, of Saidman, Sterne, Kessler & Goldstein (Henry N. Wixon, with them on brief), Washington, D.C., for appellant.

John H. Raubitschek, associate solicitor (Joseph F. Nakamura and Fred E. McKelvey, with him on brief), PTO, for appellee.

Before Smith, Newman, and Bissell, circuit judges.

Smith, J.

This appeal is from the decision of the Patent and Trademark Office (PTO) Board of Patent Appeals and Interferences (board) affirming the rejection of all remaining claims in appellant's application for a patent, serial No. 188,735, entitled "Immunoassay Utilizing Monoclonal High Affinity IgM Antibodies," which was filed September 19, 1980.[1] The rejection under 35 U.S.C. §112, first paragraph, is based on the grounds that appellant's written specification would not enable a person skilled in the art to make the monoclonal antibodies that are needed to practice the claimed invention without undue experimentation. We reverse.

I. *Issue*

The only issue on appeal is whether the board erred, as a matter of law, by sustaining the examiner's rejection for lack of enablement under 35 U.S.C. §112, first paragraph, of all remaining claims in appellants' patent application, serial No. 188,735.

II. *Background*

A. *The Art.*

The claimed invention involves immunoassay methods for the detection of hepatitis B surface antigen by using high-affinity monoclonal antibodies of the IgM isotype. *Antibodies* are a class of proteins (immunoglobulins) that help defend the body against invaders such as viruses and bacteria. An antibody has the potential to bind tightly to another molecule, which molecule is called an antigen. The body has the ability to make millions of different antibodies that bind to different antigens. However, it is only after exposure of an antigen that a complicated *immune response* leads to the production of antibodies against that antigen. For example, on the surface of hepatitis B virus particles there is a large protein called *hepatitis B surface antigen* (HBsAg). As its name implies, it is capable of serving as an antigen. During a hepatitis B infection (or when purified HBsAg is injected experimentally), the body begins to make antibodies that bind tightly and specifically to HBsAg. Such antibodies can be used as reagents for sensitive diagnostic tests (*e.g.*, to detect hepatitis B virus in blood and other tissues, a purpose of the claimed invention). A method for detecting or measuring antigens by using antibodies as reagents is called an *immunoassay*.

Normally, many different antibodies are produced against each antigen. One reason for this diversity is that different antibodies are produced that bind to different regions (determinants) of a large antigen molecule such as HBsAg. In addition, different antibodies may be produced that bind to the same determinant. These usually differ in the tightness with which they bind to the determinant. *Affinity* is a quantitative measure of the strength of antibody-antigen binding. Usually an antibody with a higher affinity for an antigen will be more useful for immunological diagnostic tests than one with a lower affinity. Another source of heterogeneity is that there are several immunoglobulin classes or *isotypes*. Immunoglobulin G (IgG) is the most common isotype in serum. Another isotype, immunoglobulin M (IgM), is prominent early in the immune response. IgM molecules are larger than IgG molecules, and have 10 antigen-binding sites instead of the 2 that are present in IgG. Most immunoassay methods use IgG, but the claimed invention uses only IgM antibodies.

For commercial applications there are many disadvantages to using antibodies from serum. Serum contains a complex mixture of antibodies against the antigen of interest within a much larger pool of antibodies directed at other antigens. There are available only in a limited supply that ends when the donor dies. The goal of monoclonal antibody technology is to produce an unlimited supply of a single purified antibody.

The blood cells that make antibodies are *lymphocytes*. Each lymphocyte makes only one kind of antibody. During an immune response, lymphocytes exposed to their particular antigen divide and mature. Each produces a *clone* of identical daughter cells, all of which secrete the same antibody. Clones of lymphocytes, all derived from a single lymphocyte, could provide a source of a single homogeneous antibody. However, lymphocytes do not survive for long outside of the body in cell culture.

Hybridoma technology provides a way to obtain large numbers of cells that all produce the same antibody. This method takes advantage of the properties of *myeloma* cells derived from a tumor of the immune system. The cancerous myeloma cells can divide indefinitely in vitro. They also have the potential ability to secrete antibodies. By appropriate experimental manipulations, a myeloma cell can be made to fuse with a lymphocyte to produce a single hybrid cell (hence, a hybridoma) that contains the genetic material of both cells. The hybridoma secretes the same antibody that was made by its parent lymphocyte, but acquires the capability of the myeloma cell to divide and grow indefinitely in cell culture. Antibodies produced by a clone of hybridoma cells (i.e., by hybridoma

[1] *In re Wands*, Appeal No. 673-76 (Bd. Pat. App. & Int. Dec. 30, 1986).

cells that are all progeny of a single cell) are called monoclonal antibodies.[2]

B. *The Claimed Invention.*

The claimed invention involves methods for the immunoassay of HBsAg by using high-affinity monoclonal IgM antibodies. Jack R. Wands and Vincent R. Zurawski, Jr., two of the three coinventors of the present application, disclosed methods for producing monoclonal antibodies against HBsAg in United States patent No. 4,271,145 (the '145 patent), entitled "Process for Producing Antibodies to Hepatitis Virus and Cell Lines Therefor," which patent issued on June 2, 1981. The '145 patent is incorporated by reference into the application on appeal. The specification of the '145 patent teaches a procedure for immunizing mice against HBsAg, and the use of lymphocytes from these mice to produce hybridomas that secrete monoclonal antibodies specific for HBsAg. The '145 patent discloses that this procedure yields both IgG and IgM antibodies with high-affinity binding to HBsAg. For the stated purpose of complying with the best mode requirement of 35 U.S.C. §112, first paragraph, a hybridoma cell line that secretes IgM antibodies against HBsAg (the 1F8 cell line) was deposited at the American Type Culture Collection, a recognized cell depository, and became available to the public when the '145 patent issued.

The application on appeal claims methods for immunoassay of HBsAg using monoclonal antibodies such as those described in the '145 patent. Most immunoassay methods have used monoclonal antibodies of the IgG isotype. IgM antibodies were disfavored in the prior art because of their sensitivity to reducing agents and their tendency to self-aggregate and precipitate. Appellants found that their monoclonal IgM antibodies could be used for immunoassay of HbsAg with unexpectedly high sensitivity and specificity. Claims 1, 3, 7, 8, 14, and 15 are drawn to methods for the immunoassay of HBsAg using high-affinity IgM monoclonal antibodies. Claims 19 and 25-27 are for chemically modified (*e.g.*, radioactively labeled) monoclonal IgM antibodies used in the assays. The broadest method claim reads:

> 1. An immunoassay method utilizing an antibody to assay for a substance comprising hepatitis B-surface antigen (HBsAg) determinants which comprises the steps of:
>
> contacting a test sample containing said substance comprising HBsAg determinants with said antibody; and
>
> determining the presence of said substance in said sample;
>
> wherein said antibody is a monoclonal high affinity IgM antibody having a binding affinity constant for said HBsAg determinants of at least 10^9 M^{-1}.

Certain claims were rejected under 35 U.S.C. §103; these rejections have not been appealed. Remaining claims 1, 3, 7, 8, 14, 15, 19, and 25-27 were rejected under 35 U.S.C. §112, first paragraph, on the grounds that the disclosure would not enable a person skilled in the art to make and use the invention without undue experimentation. The rejection is directed solely to whether the specification enables one skilled in the art to make the monoclonal antibodies that are needed to practice the invention. The position of the PTO is that data presented by Wands show that the production of high-affinity IgM anti-HBsAg antibodies is unpredictable and unreliable, so that it would require undue experimentation for one skilled in the art to make the antibodies.

III. *Analysis*

A. *Enablement by Deposit of Micro-organisms and Cell Lines.*

The first paragraph of 35 U.S.C. §112 requires that the specification of a patent must enable a person skilled in the art to make and use the claimed invention. "Patents * * * are written to enable those skilled in the art to practice the invention."[3] A patent need not disclose what is well known in the art.[4] Although we review underlying facts found by the board under a "clearly erroneous" standard,[5] we review enablement as a question of law.[6]

Where an invention depends on the use of living materials such as microorganisms or

[2] For a concise description of monoclonal antibodies and their use in immunoassay see *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1368-71, 231 USPQ 81, 82-83 (Fed. Cir. 1986), *cert. denied*, 107 S.Ct. 1606 (1987).

[3] *W.L. Gore & Assocs., Inc. v. Garlock, Inc.*, 721 F.2d 1540, 1556, 220 USPQ 303, 315 (Fed. Cir. 1983), *cert. denied*, 469 U.S. 851 (1984).

[4] *Lindemann Maschinenfabrik GMBH v. American Hoist & Derrick Co.*, 730 F.2d 1452, 1463, 221 USPQ 481, 489 (Fed. Cir. 1984).

[5] *Coleman v. Dines*, 754 F.2d 353, 356, 224 USPQ 857, 859 (Fed. Cir. 1985).

[6] *Moleculon Research Corp. v. CBS, Inc.*, 793 F.2d 1261, 1268, 229 USPQ 805, 810 (Fed. Cir. 1986), *cert. denied*, 107 S.Ct. 875 (1987); *Raytheon Co. v. Roper Corp.*, 724 F.2d 951, 960 n.6, 220 USPQ 592, 599 n.6 (Fed. Cir. 1983), *cert. denied*, 469 U.S. 835 [225 USPQ 232] (1984).

cultured cells, it may be impossible to enable the public to make the invention (i.e., to obtain these living materials) solely by means of a written disclosure. One means that has been developed for complying with the enablement requirement is to deposit the living materials in cell depositories which will distribute samples to the public who wish to practice the invention after the patent issues.[7] Administrative guidelines and judicial decisions have clarified the conditions under which a deposit of organisms can satisfy the requirements of section 112.[8] A deposit has been held necessary for enablement where the starting materials (i.e., the living cells used to practice the invention, or cells from which the required cells can be produced) are not readily available to the public.[9] Even when starting materials are available, a deposit has been necessary where it would require undue experimentation to make the cells of the invention from the starting materials.[10]

In addition to satisfying the enablement requirement, deposit of organisms also can be used to establish the filing date of the application as the prima facie date of invention,[11] and to satisfy the requirement under 35 U.S.C. §114 that the PTO be guaranteed access to the invention during pendency of the application.[12] Although a deposit may serve these purposes, we recognized, in *In re Lundak*,[13] that these purposes, nevertheless, may be met in ways other than by making a deposit.

A deposit also may satisfy the best mode requirement of section 112, first paragraph, and it is for this reason that the 1F8 hybridoma was deposited in connection with the '145 patent and the current application. Wands does not challenge the statements by the examiner to the effect that, although the deposited 1F8 line enables the public to perform immunoassays with antibodies produced by that single hybridoma, the deposit does not enable the generic claims that are on appeal. The examiner rejected the claims on the grounds that the written disclosure was not enabling and that the deposit was inadequate. Since we hold that the written disclosure fully enables the claimed invention, we need not reach the question of the adequacy of deposits.

B. *Undue Experimentation.*

Although inventions involving microorganisms or other living cells often can be enabled by a deposit,[14] a deposit is not always necessary to satisfy the enablement requirement.[15] No deposit is necessary if the biological organisms can be obtained from readily available sources or derived from readily available starting materials through routine screening that does not require undue experimentation.[16] Whether the specification in an application involving living cells (here, hybridomas) is enabled without a deposit must be decided on the facts of the particular case.[17]

Appellants contend that their written specification fully enables the practice of

[7] *In re Argoudelis*, 434 F.2d 1390, 1392-93, 168 USPQ 99, 101-02 (CCPA 1970).

[8] *In re Lundak*, 773 F.2d 1216, 227 USPQ 90 (Fed. Cir. 1985); *Feldman v. Aunstrup*, 517 F.2d 1351, 186 USPQ 108 (CCPA 1975), *cert. denied*, 424 U.S. 912 [188 USPQ 720] (1976); Manual of Patent Examining Procedure (MPEP) 608.01 (p)(C) (5th ed. 1983, rev. 1987). *See generally* Hampar, *Patenting of Recombinant DNA Technology: The Deposit Requirement*, 67 J. Pat. Trademark Off. Soc'y 569 (1985).

[9] *In re Jackson*, 217 USPQ 804, 807-08 (Bd. App. 1982) (strains of a newly discovered species of bacteria isolated from nature); *Feldman*, 517 F.2d 1351, 186 USPQ 108 (uncommon fungus isolated from nature); *In re Argoudelis*, 434 F.2d at 1392, 168 USPQ at 102 (novel strain of antibiotic-producing microorganism isolated from nature); *In re Kropp*, 143 USPQ 148, 152 (Bd. App. 1959) (newly discovered microorganism isolated from soil).

[10] *Ex parte Forman*, 230 USPQ 546, 547 (Bd. Pat. App. & Int. 1986) (genetically engineered bacteria where the specification provided insufficient information about the amount of time and effort required); *In re Lundak*, 773 F.2d 1216, 227 USPQ 90 (unique cell line produced from another cell line by mutagenesis).

[11] *In re Lundak*, 773 F.2d at 1222, 227 USPQ at 95-96; *In re Feldman*, 517 F.2d at 1355, 186 USPQ at 113; *In re Argoudelis*, 434 F.2d at 1394-96, 168 USPQ at 103-04 (Baldwin, J. concurring).

[12] *In re Lundak*, 773 F.2d at 1222, 227 USPQ at 95-96; *In re Feldman*, 517 F.2d at 1354, 186 USPQ at 112.

[13] *In re Lundak*, 773 F.2d at 1222, 227 USPQ at 95-96.

[14] *In re Argoudelis*, 434 F.2d at 1393, 168 USPQ at 102.

[15] *Tabuchi v. Nubel*, 559 F.2d 1183, 194 USPQ 521 (CCPA 1977).

[16] *Id.* at 1186-87, 194 USPQ at 525; *Merck & Co. v. Chase Chem. Co.*, 273 F.Supp. 68, 77, 155 USPQ 139, 146 (D.N.J. 1967); *Guaranty Trust Co. v. Union Solvents Corp.*, 54 F.2d 400, 403-06, 12 USPQ 47, 50-53 (D. Del. 1931), *aff'd*, 61 F.2d 1041, 15 USPQ 237 (3d Cir. 1932), *cert. denied*, 288 U.S. 614 (1933); MPEP 608.01(p)(C) ("No problem exists when the microorganisms used are known and readily available to the public.").

[17] *In re Jackson*, 217 USPQ at 807; *see In re Metcalfe*, 410 F.2d 1378, 1382, 161 USPQ 789, 792 (CCPA 1969).

their claimed invention because the monoclonal antibodies needed to perform the immunoassays can be made from readily available starting materials using methods that are well known in the monoclonal antibody art. Wands states that application of these methods to make high-affinity IgM anti-HBsAg antibodies requires only routine screening, and that does not amount to undue experimentation. There is no challenge to their contention that the starting materials (i.e., mice, HBsAg antigen, and myeloma cells) are available to the public. The PTO concedes that the methods used to prepare hybridomas and to screen them for high-affinity IgM antibodies against HBsAg were either well known in the monoclonal antibody art or adequately disclosed in the '145 patent and in the current application. This is consistent with this court's recognition with respect to another patent application that methods for obtaining and screening monoclonal antibodies were well known in 1980.[18] The sole issue is whether, in this particular case, it would require undue experimentation to produce high-affinity IgM monoclonal antibodies.

Enablement is not precluded by the necessity for some experimentation such as routine screening.[19] However, experimentation needed to practice the invention must not be undue experimentation.[20] "the key word is 'undue,' not 'experimentation.' "[21]

> The determination of what constitutes undue experimentation in a given case requires the application of a standard of reasonableness, having due regard for the nature of the invention and the state of the art. Ansul Co. v. Uniroyal, Inc. [448 F.2d 872, 878-79; 169 USPQ 759, 762-63 (2d Cir. 1971), *cert. denied*, 404 U.S. 1018 [172 USPQ 257] (1972)]. The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed * * * .[22]

The term "undue experimentation" does not appear in the statute, but it is well established that enablement requires that the specification teach those in the art to make and use the invention without undue experimentation.[23] Whether undue experimentation is needed is not a single, simple factual determination, but rather is a conclusion reached by weighing many factual considerations. The board concluded that undue experimentation would be needed to practice the invention on the basis of experimental data presented by Wands. These data are not in dispute. However, Wands and the board disagree strongly on the conclusion that should be drawn from that data.

Factors to be considered in determining whether a disclosure would require undue experimentation have been summarized by the board in *Ex parte Forman*.[24] They include (1) the quantity of experimentation necessary, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims.[25]

In order to understand whether the rejection was proper, it is necessary to discuss further the methods for making specific monoclonal antibodies. The first step for making monoclonal antibodies is to immunize an animal. The '145 patent provides a detailed description of procedures for immunizing a specific strain of mice against HBsAg. Next the spleen, an organ rich in lymphocytes, is removed and the lymphocytes are separated from the other spleen cells. The lymphocytes are mixed with myeloma cells, and the mixture is treated to cause a few of the cells to fuse with each other. Hybridoma cells that secrete the desired antibodies then must be isolated from the enormous number of other cells in the mixture. This is done through a series of screening procedures.

The first step is to separate the hybridoma cells from unfused lymphocytes and myeloma cells. The cells are cultured in a medium in which all the lymphocytes and myeloma cells die, and only the hybridoma cells survive. The next step is to isolate and clone hybridomas that make antibodies that bind to the antigen of interest. Single hybridoma cells are placed in separate chambers and are allowed to grow and divide. After there are enough cells in the clone to produce sufficient quantities of antibody to analyze, the antibody is assayed to determine whether it binds to the antigen. Generally, antibodies from many clones do not bind the antigen, and these clones are discarded. However, by screening enough clones (often hundreds at a time), hybridomas may be found that secrete antibodies against the antigen of interest.

Wands used a commercially available radioimmunoassay kit to screen clones for cells that produce antibodies directed against HBsAg. In this assay the amount of radioactivity bound gives some indication of the strength of the antibody-antigen binding, but does not yield a numerical affinity constant, which must be measured using the more laborious Scatchard analysis. In order to determine which anti-HBsAg antibodies satisfy all of the limitations of appellants' claims, the antibodies require further screening to select those which have an IgM isotype and have a binding affinity constant of at least 10^9 M^{-1}.[26] The PTO does not question that the screening techniques used by Wands were well known in the monoclonal antibody art.

During prosecution Wands submitted a declaration under 37 C.F.R §1.132 providing information about all of the hybridomas that appellants had produced before filing the patent application. The first four fusions were unsuccessful and produced no hybridomas. The next six fusion experiments all produced hybridomas that made antibodies specific for HBsAg. Antibodies that bound at least 10,000 cpm in the commercial radioimmunoassay were classified as "high binders." Using this criterion, 143 high-binding hybridomas were obtained. In the declaration, Wands stated that[27]

> It is generally accepted in the art that, among those antibodies which are binders with 50,000 cpm or higher, there is a very high likelihood that high affinity (Ka [greater than] 10^9 M^{-1}) antibodies will be found. However, high affinity antibodies can also be found among high binders of between 10,000 and 50,000, as is clearly demonstrated in the Table.

The PTO has not challenged this statement.

The declaration stated that a few of the high-binding monoclonal antibodies from two fusions were chosen for further screening. The remainder of the antibodies and the hybridomas that produced them were saved by freezing. Only nine antibodies were subjected to further analysis. Four (three from one fusion and one from another fusion) fell within the claims, that is, were IgM antibodies and had a binding affinity constant of at least 10^9 M^{-1}. Of the remaining five antibodies, three were found to be IgG, while the other two were IgM for which the affinity constants were not measured (although both showed binding well above 50,000 cpm).

Apparently none of the frozen cell lines received any further analysis. The declaration explains that after useful high-affinity IgM monoclonal antibodies to HBsAg had been found, it was considered unnecessary to return to the stored antibodies to screen for more IgMs. Wands says that the existence of the stored hybridomas was disclosed to the PTO to comply with the requirement under 37 C.F.R. §1.56 that applicants fully disclose all of their relevant data, and not just favorable results.[28] How these stored hybridomas are viewed is central to the positions of the parties.

The position of the board emphasizes the fact that since the stored cell lines were not completely tested, there is no proof that any of them are IgM antibodies with a binding affinity constant of at least 10^9 M^{-1}. Thus, only 4 out of 143 hybridomas, or 2.8 percent, were *proved* to fall within the claims. Furthermore, antibodies that were proved to be high-affinity IgM came from only 2 of 10 fusion experiments. These statistics are viewed by the board as evidence that appellants' methods were not predictable or reproducible. The board concludes that Wands' low rate of demonstrated success shows that a person skilled in the art would have to

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[18] *Hybritech*, 802 F.2d at 1384, 231 USPQ at 94.

[19] *Id.*; *Atlas Powder Co. v. E.I. DuPont De Nemours & Co.*, 750 F.2d 1569, 1576, 224 USPQ 409, 413 (Fed. Cir. 1984); *In re Angstadt*, 537 F.2d at 502-504, 190 USPQ at 218; *In re Geerdes*, 491 F.2d 1260, 1265, 180 USPQ 789, 793 (CCPA 1974); *Mineral Separation, Ltd. v. Hyde*, 242 U.S. 261, 270-71 (1916).

[20] *Hybritech*, 802 F.2d at 1384, 231 USPQ at 94; *W.L. Gore*, 721 F.2d at 1557, 220 USPQ at 316; *In re Colianni*, 561 F.2d 220, 224, 195 USPQ 150, 153 (CCPA 1977) (Miller, J., concurring).

[21] *In re Angstadt*, 537 F.2d at 504, 190 USPQ at 219.

[22] *In re Jackson*, 217 USPQ at 807.

[23] *See Hybritech*, 802 F.2d at 1384, 231 USPQ at 94; *Atlas Powder*, 750 F.2d at 1576, 224 USPQ at 413.

[24] *Ex parte Forman*, 230 USPQ at 547.

[25] *Id.*; *see In re Colianni*, 561 F.2d at 224, 195 USPQ at 153 (Miller, J., concurring); *In re Rainer*, 347 F.2d 574, 577, 146 USPQ 218, 221 (CCPA 1965).

[26] The examiner, the board, and Wands all point out that, technically, the strength of antibody-HBsAg binding is measured as *avidity*, which takes into account multiple determinants on the HBsAg molecule, rather than affinity. Nevertheless, despite this correction, all parties then continued to use the term "affinity." We will use the terminology of the parties. Following the usage of the parties, we will also use the term "high-affinity" as essentially synonymous with "having a binding affinity constant of at least 10^9 M^{-1}."

[27] A table in the declaration presented the binding data for antibodies from every cell line. Values ranged from 13,867 to 125,204 cpm, and a substantial proportion of the antibodies showed binding greater than 50,000 cpm. In confirmation of Dr. Wand's statement, two antibodies with binding less than 25,000 cpm were found to have affinity constants greater than 10^9 M^{-1}.

[28] *See Rohm & Haas Co. v. Crystal Chem. Co.*, 722 F.2d 1556, 220 USQ 98 (Fed. Cir. 1983).

engage in undue experimentation in order to make antibodies that fall within the claims.

Wands views the data quite differently. Only nine hybridomas were actually analyzed beyond the initial screening for HBsAg binding. Of these, four produced antibodies that fell within the claims, a respectable 44 percent rate of success. (Furthermore, since the two additional IgM antibodies for which the affinity constants were never measured showed binding in excess of 50,000 cpm, it is likely that these also fall within the claims.) Wands argues that the remaining 134 unanalyzed, stored cell lines should not be written off as failures. Instead, if anything, they represent partial success. Each of the stored hybridomas had been shown to produce a high-binding antibody specific for HBsAg. Many of these antibodies showed binding above 50,000 cpm and are thus highly likely to have a binding affinity constant of at least 10^9 M^{-1}. Extrapolating from the nine hybridomas that were screened for isotype (and from what is well known in the monoclonal antibody art about isotype frequency), it is reasonable to assume that the stored cells include some that produce IgM. Thus, if the 134 incompletely analyzed cell lines are considered at all, they provide some support (albeit without rigorous proof) to the view that hybridomas falling within the claims are not so rare that undue experimentation would be needed to make them.

The first four fusion attempts were failures, while high-binding antibodies were produced in the next six fusions. Appellants contend that the initial failures occurred because they had not yet learned to fuse cells successfully. Once they became skilled in the art, they invariably obtained numerous hybridomas that made high-binding antibodies against HBsAg and, in each fusion where they determined isotype and binding affinity they obtained hybridomas that fell within the claims.

Wands also submitted a second declaration under 37 C.F.R. §1.132 stating that after the patent application was submitted they performed an eleventh fusion experiment and obtained another hybridoma that made a high-affinity IgM anti-HBsAg antibody. No information was provided about the number of clones screened in that experiment. The board determined that, because there was no indication as to the number of hybridomas screened, this declaration had very little value. While we agree that it would have been preferable if Wands had included this information, the declaration does show that when appellants repeated their procedures they again obtained a hybridoma that produced an antibody that fit all of the limitations of their claims.

[1] We conclude that the board's interpretation of the data is erroneous. It is strained and unduly harsh to classify the stored cell lines (each of which was proved to make high-binding antibodies against HBsAg) as failures demonstrating that Wands' methods are unpredictable or unreliable.[29] At worst, they prove nothing at all about the probability of success, and merely show that appellants were prudent in not discarding cells that might someday prove useful. At best, they show that high-binding antibodies, the starting materials for IgM screening and Scatchard analysis, can be produced in large numbers. The PTO's position leads to the absurd conclusion that the more hybridomas an applicant makes and saves without testing the less predictable the applicant's results become. Furthermore, Wands' explanation that the first four attempts at cell fusion failed only because they had not yet learned to perform fusions properly is reasonable in view of the fact that the next six fusions were all successful. The record indicates that cell fusion is a technique that is well known to those of ordinary skill in the monoclonal antibody art, and there has been no claim that the fusion step should be more difficult or unreliable where the antigen is HBsAg than it would be for other antigens.

[2] When Wands' data is interpreted in a reasonable manner, analysis considering the factors enumerated in *Ex parte Forman* leads to the conclusion that undue experimentation would not be required to practice the invention. Wands' disclosure provides considerable direction and guidance on how to practice their invention and presents working examples. There was a high level of skill in the art at the time when the application was filed, and all of the methods needed to practice the invention were well known.

The nature of monoclonal antibody technology is that it involves screening hybridomas to determine which ones secrete antibody with desired characteristics. Practitioners of this art are prepared to screen negative hybridomas in order to find one that makes the desired antibody. No evidence was presented by either party on how many hybridomas would be viewed by those in the art as requiring undue experimentation to screen. However, it seems unlikely that undue experimentation would be defined in terms of the number of hybridomas that were never screened. Furthermore, in the monoclonal antibody art it appears that an "experiment" is not simply the screening of a single hybridoma, but is rather the entire attempt to make a monoclonal antibody against a particular antigen. This process entails immunizing animals, fusing lymphocytes from the immunized animals with myeloma cells to make hybridomas, cloning the hybridomas, and screening the antibodies produced by the hybridomas for the desired characteristics. Wands carried out this entire procedure three times, and was successful each time in making at least one antibody that satisfied all of the claim limitations. Reasonably interpreted, Wands' record indicates that, in the production of high-affinity IgM antibodies against HBsAG, the amount of effort needed to obtain such antibodies is not excessive. Wands' evidence thus effectively rebuts the examiner's challenge to the enablement of their disclosure.[30]

[29] Even if we were to accept the PTO's 2.8% success rate, we would not be required to reach a conclusion of undue experimentation. Such a determination must be made in view of the circumstances of each case and cannot be made solely by reference to a particular numerical cutoff.

[30] *In re Strahilevitz*, 668 F.2d 1229, 1232, 212 USPQ 561, 563 (CCPA 1982).

IV. *Conclusion*

Considering all of the factors, we conclude that it would not require undue experimentation to obtain antibodies needed to practice the claimed invention. Accordingly, the rejection of Wands' claims for lack of enablement under 35 U.S.C. §112, first paragraph, is reversed.

REVERSED

Newman, J., concurring in part, dissenting in part.

A

I concur in the court's holding that additional samples of hybridoma cell lines that produce these high-affinity IgM monoclonal antibodies need not be deposited. This invention, as described by Wands, is not a selection of a few rare cells from many possible cells. To the contrary, Wands states that all monoclonally produced IgM antibodies to hepatitis B surface antigen have the desired high avidity and other favorable properties, and that all are readily preparable by now-standard techniques.

Wands states that his United States Patent No. 4,271,145 describes fully operable techniques, and is distinguished from his first four failed experiments that are referred to in the Rule 132 affidavit. Wands argues that these biotechnological mechanisms are relatively well understood and that the preparations can be routinely duplicated by those of skill in this art, as in *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1380, 231 USPQ 81, 94 (Fed. Cir. 1986), *cert. denied*, 107 S.Ct. 1606 (1987). I agree that it is not necessary that there be a deposit of multiple exemplars of a cell system that is readily reproduced by known, specifically identified techniques.

B

I would affirm the board's holding that Wands has not complied with 35 U.S.C. §112, first paragraph, in that he has not provided data sufficient to support the breadth of his generic claims. Wands' claims on appeal include the following:

> 19. Monoclonal high affinity IgM antibodies immunoreactive with HBsAg determinants, wherein said antibodies are coupled to an insoluble solid phase, and wherein the binding affinity constant of said antibodies for said HBsAg determinants is at least 10^9 M^{-1}.
>
> 26. Monoclonal high affinity IgM antibodies immunoreactive with hepatitis B surface antigen.

Wands states that he obtained 143 "high binding monoclonal antibodies of the right specificity" in the successful fusions; although he does not state how they were determined to be high binding or of the right specificity, for Wands also states that only nine of these 143 were tested.

Of these nine, four (three from one fusion and one from another fusion) were found to have the claimed high affinity and to be of the IgM isotype. Wands states that the other five were either of a different isotype or their affinities were not determined. (This latter statement also appears to contradict his statement that all 143 were "high binding".)

Wands argues that a "success rate of four out of nine", or 44.4%, is sufficient to support claims to the entire class. The Commissioner deems the success rate to be four out of 143, or 2.8%; to which Wands responds with statistical analysis as to how unlikely it is that Wands selected the only four out of 143 that worked. Wands did not, however, prove the right point. The question is whether Wands, by testing nine out of 143 (the Commissioner points out that the randomness of the sample was not established), and finding that four out of the nine had the desired properties, has provided sufficient experimental support for the breadth of the requested claims, in the context that "experi-

ments in genetic engineering produce, at best, unpredictable results", quoting from *Ex parte Forman*, 230 USPQ 546, 547 (Bd.Pat.App. and Int. 1986).

The premise of the patent system is that an inventor, having taught the world something it didn't know, is encouraged to make the product available for public and commercial benefit, by governmental grant of the right to exclude others from practice of that which the inventor has disclosed. The boundary defining the excludable subject matter must be carefully set: it must protect the inventor, so that commercial development is encouraged; but the claims must be commensurate with the inventor's contribution. Thus the specification and claims must meet the requirements of 35 U.S.C. §112. *In re Fisher*, 427 F.2d 833, 839, 166 USPQ 18, 23-24 (CCPA 1970).

As the science of biotechnology matures the need for special accommodation, such as the deposit of cell lines or microorganisms, may diminish; but there remains the body of law and practice on the need for sufficient disclosure, including experimental data when appropriate, that reasonably support the scope of the requested claims. That law relates to the sufficiency of the description of the claimed invention, and if not satisfied by deposit, must independently meet the requirements of Section 112.

Wands is not claiming a particular, specified IgM antibody. He is claiming all such monoclonal antibodies in assay for hepatitis B surface antigen, based on his teaching that such antibodies have uniformly reproducible high avidity, free of the known disadvantages of IgM antibodies such as tendency to precipitate or aggregate. It is incumbent upon Wands to provide reasonable support for the proposed breadth of his claims. I agree with the Commissioner that four exemplars shown to have the desired properties, out of the 143, do not provide adequate support.

Wands argues that the law should not be "harsher" where routine experiments take a long time. However, what Wands is requesting is that the law be less harsh. As illustrated in extensive precedent on the question of how much experimentation is "undue", each case must be determined on its own facts. *See, e.g., W.L. Gore & Assocs., Inc. v. Garlock, Inc.*, 721 F.2d 1540, 1557, 220 USPQ 303, 316 (Fed. Cir. 1983), *cert. denied*, 469 U.S. 851 (1984); *In re Angstadt*, 537 F.2d 498, 504, 190 USPQ 214, 218 (CCPA 1976); *In re Cook*, 439 F.2d 730, 734-35, 169 USPQ 298, 302-03 (CCPA 1971).

The various criteria to be considered in determining whether undue experimentation is required are discussed in, for example, *Fields v. Conover*, 443 F.2d 1386, 170 USPQ 276 (CCPA 1971); *In re Rainer*, 347 F.2d 574, 146 USPQ 218 (CCPA 1965); *Ex parte Forman*, 230 USPQ at 547. Wands must provide sufficient data or authority to show that his results are reasonably predictable within the scope of the claimed generic invention, based on experiment and/or scientific theory. In my view he has not met this burden.

Patent and Trademark Office
Trademark Trial and Appeal Board

In re Johanna Farms Inc.

Serial No. 542,343

Decided June 30, 1988

JUDICIAL PRACTICE AND PROCEDURE

1. Procedure — Prior adjudication — In general (§410.1501)

Trademark Trial and Appeal Board's prior decision upholding examiner's refusal to register proposed mark "La Yogurt" does not preclude registration of mark pursuant to subsequent application, since applicant, by presenting survey evidence and consumer letters regarding issue of how purchasers perceive proposed mark, has demonstrated that instant factual situation is different from situation presented in prior proceeding.

TRADEMARKS AND UNFAIR TRADE PRACTICES

2. Types of marks — Non-descriptive — Particular marks (§327.0505)

Term "La Yogurt," with "yogurt" disclaimed, is registrable, since word "yogurt" is common English generic term rather than corruption or misspelling of French word for yogurt, since examining attorney failed to meet burden of showing clear evidence of generic use of mark as whole, and since evidence of record, including survey and consumer letters to applicant, demonstrates that primary significance of "La Yogurt" to majority of relevant public is that of brand name rather than generic term.

3. Registration and its effects — Federal registration — Procedure, form and content — Disclaimer (§315.0303.10)

Types of marks — Non-descriptive — Particular marks (§327.0505)

French article "La" combined with English generic term in proposed mark "La Yogurt" changes commercial impression of mark as whole and renders it registrable, since applicant's competitors would have no need to use "La Yogurt" mark as whole, other than to trade on applicant's good will, in view of applicant's disclaimer of word "yogurt."

Appeal from refusal of registration (Mark Traphagen, trademark examining attorney, Paul Fahrenkopf, managing attorney).

Related decisions: 222 USPQ 607, 223 USPQ 459.

Application of Johanna Farms Inc. for registration of trademark, serial no. 542,343, filed June 11, 1985. From decision refusing registration, applicant appeals. Reversed; Simms, Member, dissenting in separate opinion.

Lerner, David, Littenberg, Krumholz & Mentlik, Westfield, N. J., for applicant.

Before Sams, Rich, Rooney, Simms, Krugman, Cissel, Seeherman, and Hanak, members.

Krugman, Member.

An application has been filed by Johanna Farms, Inc. to register the term "LA YOGURT" ("YOGURT" disclaimed) as a trademark on the Principal Register for yogurt.[1] Applicant seeks registration pursuant to Section 2(f) of the Trademark Act based on the claim that the designation sought to be registered has acquired distinctiveness.

Registration has been refused on two grounds. First, the Examining Attorney maintains that registration herein is barred under the doctrine of stare decisis in view of a final decision rendered by the Board in connection with a prior application to register "LA YOGURT" for yogurt.[2] The Examining Attorney maintains that the relevant circumstances involved in the present case are identical to those considered in the prior application and that applicant is precluded from relitigating issues which have already been determined.

As a second ground for refusal, the Examining Attorney asserts that, even if it is determined that stare decisis does not bar registration herein, the phrase "LA YOGURT" is a generic designation, incapable of distinguishing applicant's goods from those of others; that "YOGURT" is the French generic name for the goods; that use of the French article "LA" in combination with "YOGURT" yields only an ungrammatical variation on the foreign generic term for the goods and that evidence of de facto secondary meaning cannot elevate the generic term sought to be registered to the status of a registrable trademark.

Applicant has appealed.

In view of the issues presented by this case, the oral hearing on November 17, 1987 was held before the eight members of the Trademark Trial and Appeal Board sitting, by designation of the Chairman of the Trademark Trial and Appeal Board, as an augmented panel.

Turning first to the issue of stare decisis, a brief review of the circumstances of the prior application and the Board's decision relating thereto are in order.

Applicant initially filed an application to register "LA YOGURT" as a trademark for yogurt on the Principal Register.[3] After registration was refused on the ground that the term sought to be registered was "merely the name of the goods," applicant amended its application to one seeking registration on the Supplemental Register. Eventually, registration was finally refused on the Supplemental Register on the ground that "LA YOGURT" was nothing more than the apt (generic) name of the goods and that said term, therefore, was unregistrable on the Supplemental Register. Applicant then appealed.

The Board, in deciding the appeal, noted that "yogurt" was concededly the name of the goods and that the term "la" had no significance by itself in relation to yogurt or any other product, other than as the French feminine article modifying the generic term. The Board then stated that the question to be decided was whether the entire term "LA YOGURT" was generic. If it were, the Board stated, the term could not be registered on either the Principal or Supplemental Register. If, however, "LA YOGURT" were not generic, it would be registrable on the Principal Register. In either case, the

[1] Application Serial No. 542,343 filed June 11, 1985.

[2] In re Johanna Farms, Inc., 222 USPQ 607 (TTAB 1984), reconsideration denied, 223 USPQ 459 (TTAB 1984).

[3] Application Ser. No. 171,952 filed May 25, 1978.

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Medical Sciences

Elimination of smooth muscle cells in experimental restenosis: Targeting of fibroblast growth factor receptors

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Communicated by Roger Guillemin, March 5, 1992

ABSTRACT **Factors in plasma and platelets do not fully account for the proliferation of smooth muscle cells in vascular injury, implying that additional factors are involved. Recently, we and others have observed that vascular injury regulates basic fibroblast growth factor, suggesting a further role for this pleiotropic factor. We report here that injury of rat arteries leads to an increase in fibroblast growth factor receptors in vascular smooth muscle cells. This up-regulation makes smooth muscle cells susceptible, *in vitro* and *in vivo*, to the lethal effects of a conjugate of basic fibroblast growth factor with the ribosome inactivator saporin. Saporin alone has no effect, whereas the conjugate kills proliferating, but not quiescent, smooth muscle cells *in vitro*. *In vivo*, one to three doses inhibit neointimal proliferation but have no apparent effect on the uninjured artery. Thus, the up-regulation of fibroblast growth factor receptors in vascular injury suggests new therapeutic possibilities for such refractory conditions as restenosis following balloon angioplasty.**

The accumulation of smooth muscle cells (SMCs) in the arterial intima is a well-recognized feature of atherosclerosis and is especially prominent in transplant atherosclerosis and restenosis after balloon angioplasty or coronary bypass grafting (1, 2). Numerous factors derived from macrophages, platelets, endothelial cells, and SMCs themselves have been implicated in the migration and proliferation of SMCs (3–5). Recently, a role has been proposed for basic fibroblast growth factor (bFGF), a heparin-binding, 18-kDa peptide best known for its angiogenic, neurotropic, and mesoderm-inducing effects (6). We and others have found that bFGF is mitogenic for SMCs (7) and that vascular bFGF expression declines after embryogenesis (8, 9) but increases when adult SMCs are placed in culture (10). Lindner *et al.* (11) have reported that bFGF infusions enhance the SMC proliferation that is a consequence of balloon injury to the rat carotid artery. Moreover, neutralizing antibodies to bFGF inhibited SMC DNA synthesis, at least transiently (12).

METHODS

Materials. Recombinant bFGF and recombinant saporin (SAP) were cross-linked and purified by heparin-Sepharose chromatography as described (13).

Cell Culture. SMCs were isolated by enzymatic digestion of the medial layers of rat aorta obtained from 7-week-old male Sprague–Dawley rats as described (10), to yield the characteristic multilayered hill and valley cultures with phase-dense cytoplasm and smooth muscle α_1 actin immunoreactivity (clone 1A4; Sigma). The cells were grown in medium 199 in 10% fetal bovine serum (FBS; Biofluids, Rockville, MD) without bFGF and split weekly at a 1:4 ratio. Assays for protein and DNA synthesis and cell proliferation are described in the figure legends.

Immunohistochemistry. Two polyclonal antibodies were raised in rabbits using synthetic peptide replicates of two domains deduced from the cDNA of FGFR-1 (*flg*). A sequence at the C terminus (CSSGEDSVFSHEPLPEEP) located beyond the tyrosine kinase domain was chosen for the intracellular domain as it has predicted antigenicity and 100% homology with the reported chicken (14), mouse (15), and human (16) FGFR-1. The corresponding region of FGFR-2 (*bek*), FGFR-3, and FGFR-4 has 72%, 61%, and 78% homology, respectively. Western blotting with this antiserum (R129) reveals a protein in embryonic and brain tissues that is not seen when the antiserum is preadsorbed with excess peptide immunogen. A second antiserum (R131) was raised to a peptide whose sequence (RITGEEVEVRDR) derives from the first (outermost) immunoglobulin-like loop of FGFR-1. It has 8%, 9%, and 18% homology with the corresponding regions of FGFR-2, FGFR-3, and FGFR-4, respectively, and gives similar results in immunostaining as the antibody to the intracellular domain.

The immunostaining was performed as described (17). Briefly, formalin-fixed, 6-μm, paraffin sections of rat carotid arteries were etched with hyaluronidase, blocked, and stained by the indirect alkaline phosphatase method. Nonimmune serum and peptide adsorption, and virtual absence of stain in A431 cells lacking FGFRs, served as negative controls. Staining of rat brain served as positive control.

Binding Assays. Membranes were prepared from normal 7-week-old rat aortas and aortas at 24 and 48 h after balloon injury (see below), as described (18). Protein levels were determined using the Pierce protein assay according to manufacturer's instructions with bovine serum albumin as a standard. Samples containing 20, 40, 60, and 80 μg of protein in 100 μl of 20 mM Hepes (pH 7.4), 1 μg of leupeptin per ml, 2 mM EDTA, 20 kallikrein inhibitor units of aprotinin per ml, 1 mM phenylmethylsulfonyl fluoride, 250 mM sucrose, and 0.2% bovine serum albumin (HB) containing 200 pM of ^{125}I-labeled bFGF were incubated in a 1.5-ml microcentrifuge tube for 30 min at 23°C, 1.0 ml of ice-cold HB/2 M NaCl was added, samples were centrifuged at 16,000 × *g*, supernatant was aspirated, and 1.0 ml of ice-cold HB was added, followed by centrifugation and aspiration of the supernatant. The tubes containing the pellets were then assayed for radioactivity in a γ counter. Background cpm obtained in the absence of membrane protein were ≤20% of the total cpm and were subtracted from the samples. Specific binding to high-affinity receptors was determined by first displacing the radiolabeled ligand from low-affinity sites with a 200-fold molar excess of

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Abbreviations: SMC, smooth muscle cell; SAP, saporin; FGF, fibroblast growth factor; bFGF, basic FGF; FGFR, FGF receptor; FBS, fetal bovine serum; FGF-SAP, bFGF-SAP conjugate.

†To whom reprint requests should be addressed.

bFGF. Binding was normalized to the amount of ^{125}I cpm bound per mg of membrane protein.

Balloon Injury. Under a protocol approved by the National Heart, Lung, and Blood Institute Animal Care and Use Committee, 8-week-old Sprague–Dawley rats were anesthetized with 75 mg of pentobarbital per kg i.p., and the left common carotid artery was cannulated with a 2 Fr Fogarty embolectomy catheter, inflated with 0.05 ml of saline and passed three times up and down the internal carotid artery to produce a distending, deendothelializing injury. At the indi-

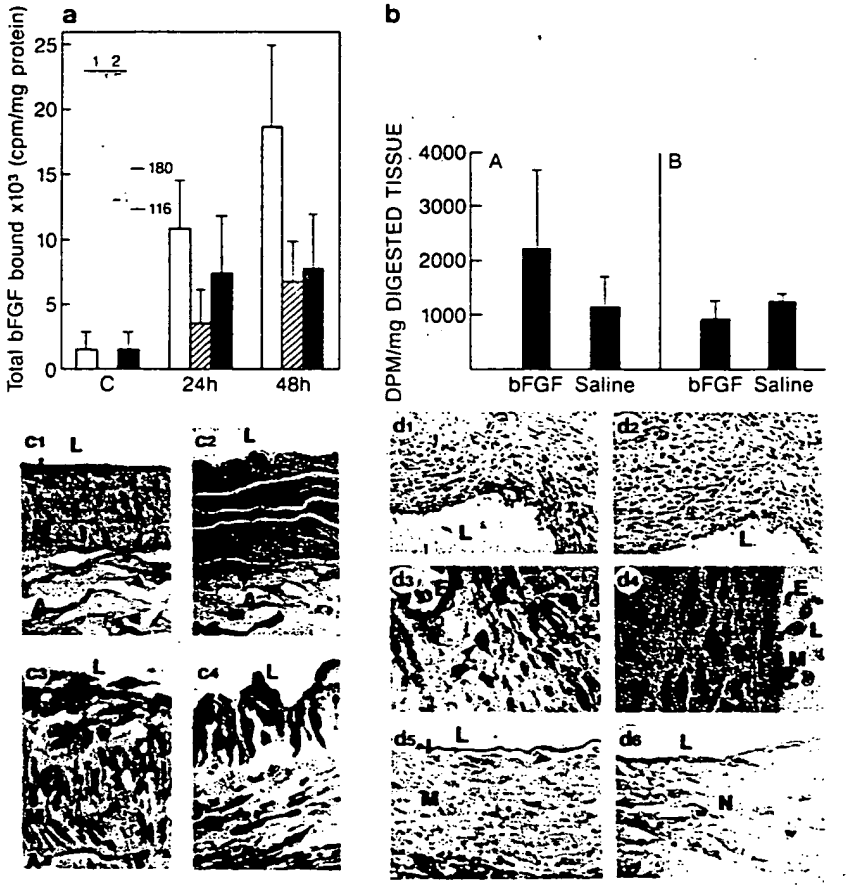

FIG. 1. FGFRs are increased by balloon injury. (*a*) Detectable binding of bFGF by membranes from balloon-injured adult arteries. Open bars, total cpm bound; hatched bars, nonspecific cpm; solid bars, specific cpm. The graph and standard deviations represent cpm determined twice from the four different protein concentrations, with each point assayed in triplicate. (*Inset*) Immunoblotting indicates a FGFR in injured, but not in normal, arteries. Normal (lane 1) and 48-h balloon-injured (lane 2) rat carotid arteries were homogenized and supernates were boiled in Laemmli buffer with 2-mercaptoethanol prior to SDS/PAGE, transfer to nitrocellulose, and blotting with anti-FGFR-1 (R131). (*b*) Repeated injections of bFGF increase thymidine incorporation in injured tissue but not in uninjured tissue. Rats (250 g) were injected every 2 h with 2 μg of bFGF s.c. or saline for 10 h and then injected with 1 μCi of [^{3}H]thymidine i.p. per g at 46 h and sacrificed at 48 h. Tissues were subjected to digestion in Protosol (New England Nuclear) according to the manufacturer's instructions and liquid scintillation spectroscopy in Ionic-Fluor (Packard), corrected by quench curves. Despite plasma levels of 0.8 ng/ml 24 h after injection (estimated by mixing 50 ng of ^{125}I-labeled bFGF in 950 ng of unlabeled bFGF and assay of trichloroacetic acid-insoluble cpm in a γ counter), a level giving nearly maximal mitogenic effect *in vitro*, only 0.33% of the bFGF tracer bound to the heart and 42% of this was displaceable by heparin. There was no effect on thymidine incorporation in the heart (B). Similar results were obtained when heart sections were embedded in paraffin and subjected to autoradiography using NTB-2 emulsion. Labeled capillary nuclei were counted in two sections of each of four rats treated with bFGF and in two rats treated with saline. The average ($\pm$SD) number of labeled nuclei per mm^2 in the bFGF-treated rats was 11.4 $\pm$ 5.3 *vs.* 12.6 $\pm$ 4.0 for the saline-treated rats ($P > 0.1$ by two-tailed t test). In contrast (*A*), the s.c. tissue injured by repeated injections revealed more local [^{3}H]thymidine uptake when injected with bFGF than with saline. (*c*) *In vivo* autoradiographic evidence that FGFRs are expressed after vascular injury. Adult rats were subjected to balloon dilation of the carotid artery and injected i.v. 2 days later with 60 ng of either mitogenically active or heat-denatured (90°C, 1 h) ^{125}I-labeled bFGF (10^5 cpm/ng iodinated as described) with or without 10 μg of unlabeled bFGF; this was followed 1 h later by i.v. heparin (165 units/kg), anesthesia by pentobarbital (75 mg/kg), and killing by perfusion with 10% formalin 1 h later. Tissues were processed for autoradiography and developed 10 days later. (*c1*) Uninjured vessel causes almost no precipitation of silver grains, indicating little or no heparin-resistant (high affinity) binding. L, lumen; M, media; A, adventitia. (*c2*) Binding of bFGF 48 h after balloon injury. (*c3*) At day 4, bFGF binds preferentially to cells in neointima (N). (*c4*) Competition by excess unlabeled bFGF (no grains over neointima). (*c1*, $\times$280; *c2–4*, $\times$395.) (*d*) Immunocytochemical localization of a FGFR after vascular injury. (*d1*) Rat aorta, 14 days after deendothelialization and dilation, stained with an antibody (R129) to an intracellular domain of FGFR-1/*flg*. Many of the proliferating SMCs exhibit the brown immunoperoxidase reaction product. L, lumen. (Methyl green counterstain; $\times$140.) (*d2*) Injured segment of same rat stained with an antibody (R131) to an extracellular domain of FGFR-1/*flg*. ($\times$140.) The similarity of the immunohistological results using antisera directed against different peptides deduced from the same receptor cDNA, the Western analysis (*a*), and the lack of stain using peptide-adsorbed (*d6*) or nonimmune serum indicate the specificity of the assay. (*d3* and *d4*) Same slides at higher power. ($\times$720.) Brown (immunoperoxidase-positive) SMCs are indicated by arrowheads. Monocyte (M) is immunoreactive but most erythrocytes (E) show only background staining. The normal segment shows little immunoreactivity of the medial layer with R131. (*d5*) Near absence of stain for the *flg* gene product. (*d6*) Normal rabbit serum is unreactive with injured vessels (or normal vessels, not shown). (*d3–d6*, $\times$395.)

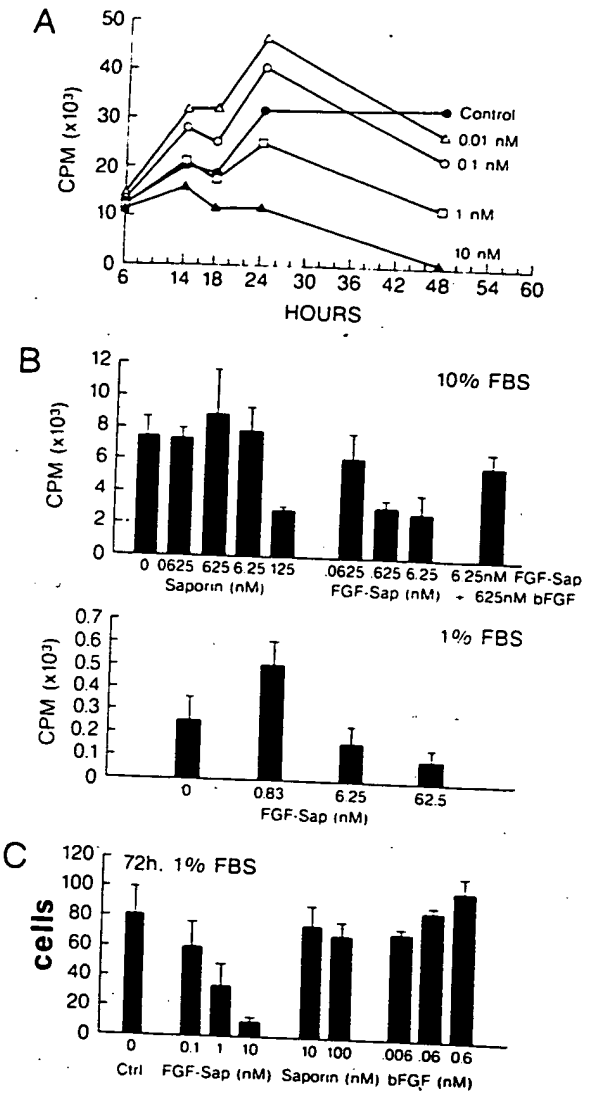

FIG. 2. Effects of FGF-SAP on protein and DNA synthesis and cell number. (*A*) Time course of effect of various concentrations of FGF-SAP on protein synthesis by SMCs. Rat aortic SMCs from eighth passage were plated at 30,000 cells per cm² in 10% FBS in leucine-free M199. At 20 h, cells received 0.01–10 nM FGF-SAP. At 14, 18, or 24 h, cells were pulsed for 2 h and assayed by liquid scintillation spectroscopy. For clarity, means are shown without SD if ≤10% of means. Also omitted are the data with 10 and 100 nM SAP, which did not differ from controls. The experiment was repeated twice with rabbit aortic SMCs, with similar results. (*B*) Inhibition of DNA synthesis by FGF-SAP, but not SAP or FGF-SAP competitively inhibited by bFGF, in rat aortic SMCs. Cells were plated in M199/1% FBS at 10,000 per cm² and exposed to additives and [³H]thymidine at 24 h, for 24 h more. Bars indicate means ± SD of triplicate wells. In 10% FBS (upper panel) no initial enhancement of DNA synthesis was noted even at low doses of FGF-SAP. However, in 1% FBS (lower panel) there is an initial increase in DNA synthesis with low (but not high) concentrations of FGF-SAP. DNA synthesis decreases further by 48 and 76 h (not shown). (*C*) Killing of SMCs by FGF-SAP. Cells were plated at 10,000 per cm² in 1% FBS/M199 for 24 h and then exposed to the indicated concentrations of FGF-SAP, SAP, or bFGF for 72 h, at which time cells excluding trypan blue were counted in three randomly chosen fields of 1.2 mm² each.

ted times the rats were treated with bFGF, SAP, saline, or GF-SAP and, at designated intervals, anesthetized and crificed {in some cases 1 h after injection of 300 μCi of H]thymidine i.p. (1 Ci = 37 GBq)} by infusion f formalin.

The percentage of cells synthesizing DNA was determined autoradiographically using Kodak NTB-2 emulsion as per manufacturer's instructions. For measurements of neointimal thickness, regions in the middle of the carotid artery, which are not reendothelialized by 10 days after balloon injury, were measured using a Zeiss Videoplan planimetry system. Five or six sections from each artery were coded for "blinded" analysis. The inter-rater reproducibility was 92%.

RESULTS

Although specific bFGF binding was readily detectable in brain and embryo tissues in our control studies, we were unable ($n > 8$) to detect such binding in membranes prepared from normal vessels. However, when membranes were prepared from tissues collected 24 h after balloon injury, there was substantial specific binding of bFGF (Fig. 1*a*) that was not displaceable by exogenous heparin, indicating that it is the high-affinity receptor. Because these tissues yield faint, diffuse cross-linking products with radiolabeled bFGF (not shown), the presence of a high-affinity receptor was confirmed by Western blotting. As shown in Fig. 1*a Inset*, the monospecific antibody R131, which is raised against a sequence spliced out of the putative secreted forms of the receptor, shows virtually no bands in normal vessels but the expected characteristic bands of 130 and 160 kDa in injured vessels.

Recognizing that these assays do not provide information on receptor function, we sought to determine whether the apparent increase in FGFRs after injury correlated with an increased responsiveness of its target cells. Because Whalen *et al.* (19) and Majack *et al.* (20) have demonstrated that the infusion of bFGF has little, if any, effect on the mitotic state of the endothelium but Cuevas *et al.* (21) have described a hypotensive effect, suggesting that FGFRs may be present, we examined whether, in our hands, an i.v. injection of bFGF (8 μg/kg) could alter DNA synthesis in the vasculature. As shown in Fig. 1*b*, bFGF was ineffective, as monitored by autoradiography after a [³H]thymidine pulse or by liquid scintillation spectroscopy of the tissue digest. In contrast, if the tissue is injured, cells localized at the lesion respond to i.v. bFGF with an increase in DNA synthesis. Accordingly, the i.v. administration of bFGF causes an accumulation of neointima (compared to injections of saline or heat-denatured FGF) following balloon injury to the rat carotid artery but no increase in the uninjured carotid (unpublished data).

Because the vessel wall is a heterogeneous mix of endothelial cells and SMCs and the adventitial layer contains fibroblasts and nerves, the locus of increased binding and receptor expression was further evaluated. Microscopic analysis of the autoradiograms reveals silver grains over the proliferating SMCs of the injured vessel (Fig. 1*c*), confirming the immunoreactivity pattern in the SMCs of the injured arteries (Fig. 1*d*). Thus, injury to the vasculature induces the expression of FGFRs.

Recognizing that the activities of bFGF appear to be limited by the expression of its receptor, we evaluated the potential use of a receptor-specific cytocidal agent to control SMC proliferation. To this end, we examined the effects of a conjugate protein consisting of bFGF and SAP, a plant-derived enzyme that inactivates ribosomes by cleaving adenine from ribose in the 28S RNA of the 60S subunit (22).

In vitro studies (Fig. 2) reveal that, when grown in 10% serum, FGF-SAP (10 nM) inhibits protein synthesis within 14 h of its addition to cells and inhibits DNA synthesis within 24 h. Cell toxicity is detected within 24–48 h and, although this process is initiated by as little as 1 h of exposure to FGF-SAP, the presence of a 400-fold excess of bFGF prevents cell death and SAP alone is 500-fold less effective than FGF-SAP. Within 96 h of the single exposure to FGF-SAP (10 nM), only

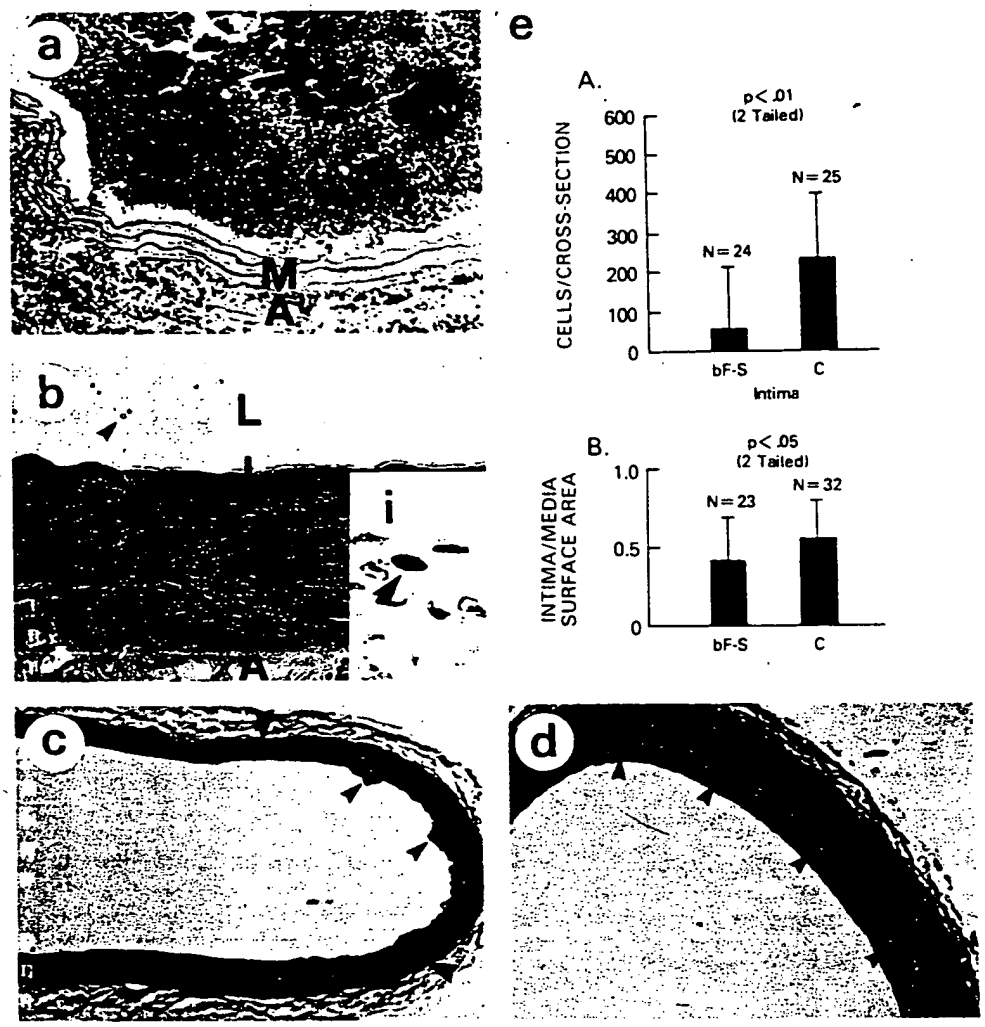

FIG. 3. After arterial injury, FGF-SAP kills proliferating SMCs and inhibits neointimal accumulation. (*a*) Twenty-four hours after balloon injury the carotid artery was occluded by proximal and distal ties and 10 μg of FGF-SAP in 20 μl of saline was instilled for 15 min, followed by release of the ligatures and sacrifice 11 days later. The medial (M) SMCs are lysed, and the layers of elastica are collapsed. The lumen is largely occluded by organizing thrombus (T). Some hemorrhage and inflammatory cells are noted in the adventitia (A). [Hemotoxylin/eosin (H&E), ×200.) SAP alone was nontoxic (not shown). (*b*) FGF-SAP was not toxic to the uninjured right carotid of a rat subjected to left carotid balloon injury and FGF-SAP, 100 μg/kg i.v. The intimal endothelium (I) and medial SMC (M) appear normal and no cells are synthesizing DNA, as illustrated by the absence of silver grains (small arrowhead) over the cell nuclei. [*Inset* (i)] An S-phase SMC from a ballooned artery treated with saline. (H&E, ×800.) (*c*) Dramatic inhibition by FGF-SAP (100 μg/kg i.v. at 24, 48, and 72 h after balloon injury) of neointimal SMC accumulation (small arrowheads): 83% less than in animals injected with SAP at 40 μg/kg (equimolar) (*d*) or saline, as determined by planimetry of neointimal areas normalized to medial areas (two-tailed unpaired *t* test, $P = 0.004$). However, toxicity was indicated by an 8% weight loss and by a few areas of medial SMC lysis (large arrowheads in *c*). For this reason, lower doses were tested and gave the results graphed in *e*. (*e*) Effects of lower doses of FGF-SAP on injured arteries. (*A*) Intimal SMCs from the central (nonendothelialized) segments of carotid artery from rats treated 24 h after balloon injury with a 15-min local application of 1 μg of FGF-SAP (bF-S) or saline (C), followed by sacrifice 10 days later. (*B*) Intimal/medial surface area ratios of rats given a single dose of 75 μg of FGF-SAP per kg or 40 μg of SAP per kg i.v. (an equimolar dose) 24 h after carotid balloon injury and sacrificed 12 days later. The *t* test for unpaired samples was used.

10% of cells remain viable, as indicated by exclusion of trypan blue. Moreover, 10 nM FGF-SAP had no effect in nonproliferating SMCs (not shown).

It is particularly remarkable that the initial response to low concentrations of FGF-SAP (0.1–1 nM) includes an increase in protein and DNA synthesis (Fig. 2). Although the reason for this effect is not clear, we have attributed this biphasic response to the different potencies and different mechanisms of action of FGF (signal transduction) and SAP (enzymatic action).

We tested FGF-SAP in an *in vivo* model where we had observed increased FGFR expression (Fig. 3). Under these conditions, when FGF-SAP (1–10 μg/kg) is locally applied, there is a concomitant death of most medial SMCs and a 75% reduction in neointimal cell number 14 days after injury (Fig. 3*a*). Rats given 100 μg/kg i.v. at 24, 48, and 72 h after injury had 83% less neointimal accumulation at 7 days than SAP- or saline-treated controls (Fig. 3 *c* and *d*). However, there were some areas of apparent SMC death in the medial layer, implying a risk of aneurysm or rupture. The uninjured right carotids were histologically normal, but an 8% body weight loss was noted, suggesting systemic toxicity.

Rats given i.v. FGF-SAP as a single 75 μg/kg dose 24 h after balloon injury had 24% less neointimal proliferation at 14 days and there was little evidence of the necrosis, thrombosis, or inflammation seen with local infusion of FGF-SAP or with larger doses of i.v. FGF-SAP (Fig. 3*e*). Remarkably, endothelial cells, which in cell culture have fewer FGFRs

than do SMCs, are spared the cytotoxic actions of FGF-SAP (unpublished data).

DISCUSSION

In this report, we describe evidence that balloon injury to the rat carotid artery induces high-affinity receptors for bFGF in the injured loci. Using this knowledge, we reasoned that the up-regulation of receptors permits the use of ligands like bFGF to act as specific vectors to carry toxins like SAP to eliminate specific cell populations. On this basis, we propose that FGFR-mediated cytotoxicity for SMCs may provide a strategy for developing therapies based on molecular atherectomy.

SMC migration, proliferation, and secretion of matrix are the dominant cellular events in restenosis following balloon angioplasty and are also prominent features of atherosclerosis, transplant rejection, and some forms of hypertension and Kaposi sarcoma (for reviews, see refs. 1 and 2). These processes have been largely refractory to clinical therapies, perhaps due to the development of autonomous SMC clones and/or to redundancy of mitogens. Numerous plasma factors and factors derived from macrophages, platelets, endothelial cells, and SMCs themselves have been implicated in this process (for reviews, see refs. 1 and 2). bFGF is a chemoattractant and a mitogen for vascular SMCs [though reports to the contrary should also be noted (23, 24)]. We and others (11, 12, 25) have shown that infusions of bFGF enhance SMC proliferation following balloon injury, whereas neutralizing antibodies to bFGF inhibit DNA synthesis, at least initially. Since bFGF is thought to be stored in cells and in their extracellular matrix, Lindner and Reidy (12) have proposed that cellular injury releases bFGF (which lacks a signal peptide), thereby contributing to SMC proliferation by an autocrine and/or paracrine mechanism.

This implies that FGFRs are present in the normal vessel (permitting a rapid response to released bFGF) or are induced shortly after balloon injury. In fact, the distribution of receptors for bFGF in normal adult tissues has not been settled. Some investigators have reported FGFR-1/*flg* to be present in embryonic tissues but absent in adult tissues other than brain (15, 26–29), whereas others have found these transcripts to be widespread in adult tissues (30–32). Recently, four more members of this tyrosine kinase family have been described and reported to exhibit some tissue-specific adult expression (33, 34). These receptors bind acidic FGF with high affinity and all but one of them bind bFGF and probably other FGF family members as well (31). The reasons for this unparalleled degree of redundancy are not known. Multiple alternative splicing patterns have been reported (35–37). These may confer cell specificity but they also can lead to false negative results with antibody or cRNA probes. Moreover, there are several reports of transcripts that appear to encode secreted extracellular domains capable of binding bFGF and acidic FGF (35). Finally, binding of bFGF appears to be subject to post-translational regulation (38). Not surprisingly perhaps, a developmentally regulated discrepancy between FGFR-1 mRNA and binding of bFGF has been described (39). For these reasons we focused our initial studies on binding of radiolabeled bFGF. Little specific binding was detected in normal adult vessels, with embryonic tissue and normal adult brain serving as positive controls. Balloon injury led to the appearance of binding sites for bFGF within 24 h. Because such binding could conceivably be nonfunctional (e.g., reflecting binding to secreted extracellular domains of FGFRs or to heparan sulfate proteoglycans on the cell surface), we also utilized functional assays. Thus, i.v. bFGF had no effect on DNA synthesis in normal uninjured vessels (suggesting few FGFRs on the lumenal surface of endothelial cells, poor penetration to SMCs, and/or "distal" inhibition of FGF's effects) but accelerated SMC proliferation following balloon injury. Similarly, FGF-SAP killed proliferating SMCs in balloon-injured arteries but had no effect on contralateral normal arteries in the same animal. It will be important to establish optimal dosing schedules and to determine whether this treatment can prevent SMC migration and proliferation or merely cause a delay. The question of which of the many types of FGFRs is up-regulated following balloon injury will require additional experiments.

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